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Kampinga, Harm H.

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COMMENTARY

# Thermotolerance in mammalian cells

## Protein denaturation and aggregation, and stress proteins

**Harm H. Kampinga**

Department of Radiobiology, Faculty of Medicine, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands

### SUMMARY

**Cells that have been pre-exposed to thermal stress can acquire a transient resistance against the killing effect of a subsequent thermal stress. The cause for this phenomenon, called thermotolerance, seems to be an enhanced resistance of proteins against thermal denaturation and aggregation. This resistance can be expressed as an attenuation of damage formation (less initial damage) or as a better repair of the protein damage (facilitated recovery). Heat Shock (or better, Stress) Proteins (HSPs) may play a role in and even be required for thermal resistance. However, rather than stress-induced enhanced synthesis and elevated total levels of HSPs per se, the concentration of, both constitutive and inducible, HSPs at and/or (re)distributed to specific subcellular sites may be the most important factor for the acquisition of thermotolerance. Specific HSPs may be involved either in damage protection or in damage repair.**

Key words: thermotolerance, protein denaturation, HSPs

### INTRODUCTION

Pre-exposure of (mammalian) cells to heat or other types of stress (e.g. sodium arsenite, diamide, ethanol, $H_2O_2$) can lead to the acquisition of a transient resistant state by the cells against the cell killing effect (clonogenic ability) of a subsequent (heat) stress. This phenomenon, known as thermotolerance (Gerner and Schneider, 1975) is distinct from stable tolerance as obtained by cloning survivors after repeated heating cycles, mutant selection or (stable) transfections, although underlying mechanisms responsible for the reduced cell killing effect of heat may share common features. Furthermore, thermotolerance also has been used to describe resistance to heat effects at the subcellular, biochemical or molecular level, although they might have no relation to thermal killing. For clarity, here *thermotolerance* (TT) will only be used to indicate an *induced transient resistance* against the *cell killing* effect of heat. Unless otherwise stated, data on mammalian cells only are discussed.

Operationally, one can distinguish three types of TT (see Henle, 1987, for details). Chronic TT (HTTc) is the development of TT during heating at relative low hyperthermic temperatures (usually below 42˚C) as a result of which no further killing beyond a certain time of exposure at that temperature is found: at that time, the HTTc cells are also resistant to the cell killing effect of higher temperatures. Acute TT (HTTa) is induced by a short exposure of cells to relative high temperatures (usually > 43˚C) followed by a period at 37˚C for TT development. Finally, exposure of cells to a variety of chemicals (Henle, 1987) followed by a drug-free period at 37˚C can also lead to TT development (chemically induced TT: ChTT). The extent of TT and the kinetics of development and decay depend on many factors as discussed in detail by Henle (1987). TT occurs in nearly all mammalian cell types, one-cell mouse embryos being the only reported exception (Muller et al. 1985).

In this commentary, I will predominantly discuss the relation between TT, protein denaturation and aggregation, and Heat Shock (or better Stress) Proteins (HSP).

### HEAT KILLING

In order to know how cells can become thermotolerant, one has to know more about the mechanisms putatively involved in heat killing. Hyperthermic treatment of mammalian cells leads to many alterations, including inactivation of the “normal” pattern of genetic expression at various levels (transcription, splicing, translation), inhibition of cell proliferation (DNA synthesis, cell division), alterations in cell morphology and cell adhesion, reduction in responses to hormones, enhancement of the effects of various drugs and of radiation (Konings, 1988; Bensaude et al. 1991; Laszlo, 1992). This implies that many cellular structures are affected after heating cells, which is indeed what has been observed (reviewed by Laszlo, 1992). This may

seem discouraging when trying to elucidate (the) critical molecular event(s) leading to the mentioned heat effects. However, all these effects may have a general denominator: proteins. The loss of structural integrity of subcellular components (and consequently the loss of cellular functions), some of which eventually may lead to cell death, may all be consequences of (nonspecific) protein denaturation. This is in accordance with the high activation energy (~600 kJ/mol) associated with the killing effect of heat (Lepock, 1987). Protein denaturation has been shown to take place in mammalian cells at hyperthermic temperatures (Lepock, 1987; Lepock et al. 1988, 1990b; Burgman and Konings, 1992).

The "fate" of partially denatured/unfolded proteins may be that they will aggregate with each other. It is known that incorrect folding of proteins can result in aggregation of proteins into so-called "inclusion bodies" as the result of severe overproduction of a protein by transfection (Pain 1987). In our studies (Burgman and Konings 1992; Burgman et al. 1992), heat-induced protein denaturation (as measured using electron spin resonance, ESR) was always paralleled by the formation of high molecular mass protein aggregates (measured by thermal gel analysis, TGA, using non-reducing SDS-PAGE). Insolubilization of various cellular proteins has been reported as a consequence of heat treatment of cells. A higher recovery of nuclear DNA polymerase a and β activity was found in nuclei isolated from heated cells (Kampinga et al. 1985). Also, a decrease in solubility of the nuclear (proto)oncogene products (myc, myb and p53 families), RNA polymerases, p68 kinase (transfected), β-galactosidase and (transfected) luciferase occurs in heat-shocked cells (Evan and Hancock, 1985; Littlewood et al. 1987; Luscher and Eisenman, 1988; Nguyen et al. 1989; Fisher et al. 1989; Dubois et al. 1991). But, what happens with these "identified" nuclear proteins probably has to be considered as part of a more general phenomenon, i.e. the widely observed overall increase in the protein mass of nuclear structures when isolated from heated cells (Roti Roti and Winward, 1978; Tomasovic et al. 1978; Kampinga et al. 1985; Warters et al. 1986; McConnell et al. 1987; Fisher et al. 1989). This increase in insolubility of proteins indicates aggregation of these, usually soluble (nuclear), proteins with the detergent-insoluble nuclear fraction. Under several conditions (including HTT), a good correlation was found between the extent and duration of this increased protein mass of isolated nuclei and thermal killing (Kampinga et al. 1989). Under conditions where proteins are stabilized, e.g. when heated in the presence of glycerol or $D_2O$ (Massicotte-Nolan et al. 1981; Lepock et al. 1988), a smaller increase in insolubility of "marker" proteins is observed (Dubois et al. 1991) and "total" protein aggregation in nuclei is reduced (Henle and Warters, 1982; Kampinga et al. 1989; Borelli, M. J., personal communication). These treatments also lead to protection at the level of cell survival (clonogenic ability: Massicotte-Nolan et al. 1981; Henle and Warters, 1982; Kampinga et al. 1989). Agents like ethanol or procaine, which can sensitize for heat killing, lower the denaturation temperature of proteins (Lepock et al. 1988; Burgman et al. unpublished), enhance insolubilization of individual proteins (Nguyen et al. 1989) and enhance the overall heat-induced protein aggregation in the nucleus (Roti Roti and Wilson, 1984; Kampinga et al. 1989). Interestingly, several HSPs often form part of the insoluble aggregates (see Bensaude et al. 1991, for review: see also below).

## TRIGGER FOR TT DEVELOPMENT

If protein denaturation and, subsequent, aggregation are indeed the most important damage induced by heat, it seems obvious to speculate that in reaction to this damage, cellular protein structures may become resistant via stabilization of their proteins against thermal denaturation and subsequent insolubilization/aggregation. Several lines of evidence indeed suggest that denaturation of proteins is the trigger for the development of TT (see e.g. Hahn and Li, 1990; Hightower, 1991). For example, many of the chemical inducers of TT are oxidizing agents that (can) cause protein damage.

## THERMOTOLERANCE: INITIAL DAMAGE VERSUS REPAIR

It is still a matter for debate as to whether TT is due to some kind of protective mechanism leading to less initial damage or due to better "repair" (in terms of recovery) of heat damage. Actually, for thermal resistance of proteins in HTTa cells both features have been observed: heat-induced protein denaturation was attenuated in membranes of HTT HeLa cells, and enzyme insolubilization was reduced (Nguyen et al. 1989; Dubois et al. 1991). However, in the same HeLa cells, the (overall) intranuclear protein aggregation was the same for tolerant and nontolerant cells; here, a more rapid disaggregation with time after heat was observed for the HTT cells (Kampinga et al. 1987, 1989). Furthermore, e.g., heat-induced inhibition of macromolecular processes like protein synthesis was sometimes found to be less in HTT cells (Mizzen and Welch, 1988), whereas in other cases the same initial inhibition, but faster recovery, was observed (Black and Subjeck, 1989; Kampinga et al. 1992a). The absence/presence of protection against initial heat damage may very well be dependent on the time span chosen for development of TT (see Laszlo, 1988a, for detailed discussion), the cellular target investigated, or the (over)expression of different HSPs in the various of cell lines used (Yost and Lindquist, 1991; see also below). Although it may not be the rule that heat-induced changes are less in TT cells, they always seem to recover faster. For the purpose of simplicity, thermal resistance of TT cells will be used further to indicate resistance at both the level of initial damage and at the level of enhanced repair.

## RESISTANCE OF STRUCTURE AND FUNCTIONS TO THERMAL DAMAGE

In thermotolerant cells many protein structures and cellular functions seem to be protected against thermal damage/inactivation (Laszlo, 1992). As already mentioned, with regard to protein denaturation and aggregation, it has

**Table 1. Comparison of the damage and resistance induced in membranes and nuclei by different thermotolerance-inducing agents (target-resistance hypothesis) in HeLa S3 cells**

| TT | $TRR_{10}$ | Damage to membranes (by agent alone) | Membrane resistance† | Damage to nuclei (by agent alone) | Nuclear resistance‡ |
|---|---|---|---|---|---|
| C | (1.0) | – | – | – | – |
| HTT | 2.3 | Yes | Yes | Yes | Yes |
| ATT | 1.8 | Yes | Yes | No | No |
| DTT | 2.5 | n.d.* | No | Yes | Yes |
| ETT | 2.3 | No | No | Yes | Yes |

HTT, ATT, DTT and ETT: thermotolerance induced by heat (H), arsenite (A), diamide (D), and ethanol (E); $TTR_{10}$: thermotolerance ratio = ratio of heating times at 45˚C necessary to reduce survival to 10% in nontolerant and thermotolerant cells.

*Not done: agent itself interferes with measurement.

†Membrane resistance, as defined by a shift in denaturation temperature as measured by ESR and TGA (Burgman and Konings, 1992; Burgman et al. 1992).

‡Nuclear resistance, as defined by extent and duration of heat-induced intranuclear protein aggregation (Kampinga et al. 1989, 1992a,b).

been observed that membrane proteins in HTT cells are resistant to protein denaturation, i.e. denaturation starts at higher temperatures (Lepock et al. 1990b; Burgman and Konings, 1992). In ChTT cells, the situation seems to be more complicated (Table 1). It was found that in cells made TT by pre-treatment with sodium arsenite (ATT), the proteins in the cellular membranes had become heat-resistant (Burgman et al. 1992a), whereas those in the nucleus had not (Kampinga et al. 1992a). On the other hand, cells made TT by diamide (DTT) or ethanol (ETT) were found to be resistant at the level of the nucleus, but not at the level of the membranes. Interestingly, thermal resistance was only found in those subcellular fractions in which the inducing agents caused damage by themselves (Burgman et al. 1992; Kampinga et al. 1992b: Table 1). Prior heating, causing protein damage throughout the cells, leads to thermal resistance in both fractions tested. The results, therefore, suggest that induction of thermal resistance is restricted to the damaged site ("target-resistance"). Resistance of one or more subcellular protein structures (individually or together) can lead to some TT.

## THERMOTOLERANCE: DO WE NEED HSPS?

Excellent reviews have been written on the putative involvement of HSPs in TT (and stable resistance) (Li and Laszlo, 1985; Lindquist and Craig, 1988; Hahn and Li, 1990; Morimoto et al. 1990; Welch, 1990: to name only a few). There seems to be ample evidence that, when HSPs are present in elevated amounts, cells become refractory to the killing effect of heat. Transfections with individual HSP genes like HSP27 (Landry et al. 1989) or HSP72 (Li et al. 1991; Angelidis et al. 1991) were shown to lead to stable resistance. On the other hand, yeast cells with a knock-out mutation in the HSP104 gene were found to have enhanced heat sensitivity and were unable to develop TT (Sanchez and Lindquist, 1990). Competitive inhibition of heat-induced HSP70 expression leads to increased thermosensitivity (Johnston and Kucey, 1988). Finally, microinjection with antibodies against HSP70 leads to reduced heat resistance (Riabowol et al. 1988). The finding that the most likely triggers for HSP synthesis are denatured/aggregated proteins (Finley et al. 1984; Lee and Hahn, 1988; Lepock et al. 1990a; Hightower, 1991; Hahn and Li, 1990; Morimoto et al. 1990) and the excellent correlations observed between development and decay of TT and HSP synthesis (Li and Werb, 1982; Hahn and Li, 1990) indeed also suggest a causal relation between HSP expression and TT. However, data showing that the use of inhibitors of protein synthesis during TT development did not (or only partially) affect TT expression in several cases (Landry and Crétien, 1983; Widelitz et al. 1986; Laszlo, 1988b; Lee and Dewey, 1988; Kampinga et al. 1992a) have challenged the necessity of newly synthesized (and thus elevated cellular levels) HSPs in TT. Recently, the results found with these inhibitors (with always the possibility of side-effects) were supported by a study in which cells with a mutated heat shock transcription factor (HSF: necessary for stress-induced heat shock gene activation via interaction with the Heat Shock Element, HSE) were found to be able to develop full thermotolerance (Smith and Yaffe, 1991).

Do all the latter data exclude the need for HSPs in TT? They might not. All stress treatments used to induce TT inhibit normal cellular functions, including protein synthesis in which constitutive HSPs are involved (Beckmann et al. 1990). Inhibition of these processes may release HSPs from their physiological functions so that they become available for stress protection! Recent data from Lee and coworkers (1991) using histidinol-resistant variants have indeed indicated that cessation of protein synthesis can lead to protection against heat killing. But even this might not be enough. HSPs also must be reallocated to be protective at the various subcellular levels. In cells exposed to heat, it is, e.g., observed that HSP72/73 are translocated to the nucleus/nucleolus and are found associated with the salt-insoluble nuclear matrix (Welch and Suhan, 1985; Ohtsuka et al. 1986; Kampinga et al. 1988, 1992a,c). Translocation to cellular membranes was observed as well when cell fractionation (but not in situ immunofluorescence) was used (Tomasovic et al. 1989; Table 2). Data from our laboratory (Table 2) show that heat resistance of proteins in cellular membrane fractions is only found under those conditions where elevated HSP72 levels are found in these membranes. This is observed even under conditions where HSP synthesis is inhibited by cycloheximide (HTT + CHX). In the case of tolerance induced by ethanol (ETT), no thermal resistance of membrane proteins or elevated HSP72 was found in this fraction. For cells made TT by arsenite (ATT), HSP72 was only found in this fraction when TT was

**Table 2. Comparison of the amount of HSP72 in cellular membranes and protein resistance at the membrane level in HeLa S3 cells: effect of inhibition of protein synthesis**

| TT | $TTR_{10}$ | Membrane resistance | HSP72 levels in membranes |
|---|---|---|---|
| C | (1.0) | − | Very low |
| HTT | 2.3 | Yes | Elevated |
| HTT + CHX | 1.6 | Yes | Elevated |
| ATT | 1.8 | Yes | Elevated |
| ATT + CHX | 1.0 | No | Very low |
| ETT | 2.3 | No | Very low |
| ETT + CHX | 1.6 | No | Very low |

HTT, ATT and ETT: thermotolerance induced by heat (H), arsenite (A) and ethanol (E), with or without cycloheximide (CHX). $TTR_{10}$: thermotolerance ratio = ratio of heating times at 45˚C necessary to reduce survival to 10% in nontolerant and thermotolerant cells; Membrane resistance, as defined by a shift in denaturation/aggregation temperature as measured by ESR and TGA (Burgman and Konings, 1992; Burgman et al. 1992); HPS levels were measured by Western blotting using anti-HSP72 (C92F3A-5 Amersham) (Burgman et al., unpublished data).


**Fig. 1.** Possible role(s) of HSPs in protection against and "repair" from heat-induced protein denaturation and aggregation; − indicates retardation; + indicates enhancement.

observed. TT as induced by arsenite is fully protein synthesis-dependent (Laszlo, 1988b; Lee and Dewey, 1988; Kampinga et al. 1992a), which finding might be related to the mechanism of protein synthesis inhibition by this agent (see Burgman et al. 1992, for detailed discussion). In the case of ATT + CHX, no resistance and no HSP72 were found in the cellular membranes. All these results might suggest that HSPs are involved in and may even be required for TT (as e.g. suggested by Sanchez and Lindquist, 1990). However, rather than HSP synthesis and elevated levels per se, the concentration of constitutive + stress-induced HSPs at specific subcellular sites may be the main determining factor for the acquisition of TT. Indications of the importance of proper localization of HSPs in order to render cells heat-resistant also come from experiments with cells overexpressing c-*myc* and v-*myc*; these cells showed "abnormal (re)distribution" of HSP70 (Koskinen et al. 1991) and were found to be more sensitive to hyperthermia in terms of heat killing (Li et al. 1990).

## HOW CAN THE VARIOUS HSPs ACTUALLY PROTECT AGAINST THERMAL PROTEIN DAMAGE?

In Fig. 1 the possibilities are summarized. HSPs could protect cells against thermal damage, either by reducing the amount of initial damage to proteins or by enabling a more rapid "repair" of the damaged structures.

Proteins encoded by the HSP70 gene family have been the most intensively investigated. The two major proteins of this gene family are HSP72 (constitutively synthesized in primates but not rodents, and strongly induced after heat stress) and HSP73 (constitutively synthesized in most cells and (slightly) heat-inducible). HSP73 was shown to facilitate the uncoating and release of clathrin triskelions from clathrin-coated vesicles (Ungewickel, 1985; Chappell et al. 1986; DeLuca-Flaherty et al. 1990) and found to be involved in protein folding of nascent polypeptides (Beckmann et al. 1990), and in the mechanism of protein translocation across intracellular membranes (Chirico et al. 1988; Deshaies et al. 1988). All these reactions seem to be ATP-dependent. Pelham (1986) proposed that one of the functions of the HSP70 proteins under stress conditions is to bind to (heat)-denatured or otherwise damaged proteins and prevent or slow down their aggregation. Our data (Table 2) indicate that HSP72 may actually even attenuate the denaturation of (membrane) proteins as measured using ESR. In addition, a role for HSP70 proteins in "dissolving" hydrophobic protein aggregates formed under stress conditions (in a reaction driven by ATP) was suggested (Pelham, 1984, 1986; Welch and Mizzen, 1988). Data from Skowyra et al. (1990) suggested that DnaK (the single prokaryotic homologue of HSP72/73) can both prevent protein aggregation and disaggregate heat-induced complexes. At present, these are also the only data showing functional recovery of a protein (RNA polymerase) from thermal denaturation. In recent experiments done in collaboration with G.C. Li (Stege et al. unpublished), using Rat-1 cells transfected with the human HSP72 gene (Li et al. 1991), however, evidence was obtained that this HSP72 plays no role in the disaggregation of heat-induced intranuclear protein aggregates. In addition HSP72 levels in the TX-100-insoluble nuclear fraction increased after heating (Kampinga et al. 1992c) in a way that could not be related to the disaggregation process. Rather, the data with the transfected Rat-1 cells pointed to a role for HSP72 in protection against the formation of heat-induced intranuclear protein aggregates. The role of HSP73 under stress conditions is still unclear. It might have a protective role or/and it might play a role in the recovery process, as seems to be suggested by the findings of Lewis and Pelham (1985). So, in eukaryotes, the two functions of DnaK might have evolved into two (or more) proteins of the HSP70 family. But, other HSPs also have to be considered. From experiments with yeast cells, Yost and Lindquist (1991) suggested that some HSPs, of 70 and 82 kDa, may be involved in protection against heat damage, whereas others (e.g. HSP104) are involved in facilitating recovery from heat damage. In a study (unpublished data) in collaboration with J. Landry, it was found that cells transfected with HSP27 (Landry et al. 1989) show a more rapid disaggregation of heat-induced intranuclear protein aggregates, suggesting a role for HSP27 in facilitated recovery from protein damage. Interestingly, Liberek and coworkers (1991) reported that the ATPase activity of DnaK is stimulated up to 50-fold by

two other heat shock proteins, DnaJ and GrpE. For mammalian cells a similar cooperative interaction of HSPs in dealing with protein damage might exist.

To summarize: overexpression of each of the individual HSPs might lead to some level of thermal resistance and to a reduction in the thermal killing effect. Overexpression of all or most HSPs after pre-treatment with heat or chemicals will thus lead to the highest achievable resistance, and indeed in all studies with transfection of individual HSP genes, the level of resistance was always found to be lower than that of the heat-induced thermotolerant cells (Landry et al. 1989; Li et al. 1991; Angelidis et al. 1991). On the other hand, enhanced expression of HSPs is not always necessary to confer heat resistance. If constitutive HSPs can be released from their functions under physiological conditions and if they can be redistributed to damaged sites this in itself might be sufficient for thermal protection and TT expression.

## WHERE DO WE GO FROM HERE?

Although there now seems to be ample evidence that protein denaturation/aggregation is the main process leading to thermal killing and that thermotolerance may be acquired via protection against or facilitated repair of this protein damage with the aid of HSPs, several points still need to be elucidated. More information is needed about individual HSPs (transfection studies) and their putative cooperative interactions in dealing with protein damage. What are the most sensitive protein structures in the cell, which are thus rate-limiting in cell killing? What is the precise mechanism of HSP gene activation and the sequence of triggering? By which mechanism are the various HSPs translocated to the different cellular compartments and how is the damage recognized? Just a few unanswered questions on the mechanism of acquired resistance that will "trigger" us to perform more studies in this fascinating field of cell biology.

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